SmyD1, a histone methyltransferase, is required for myofibril organization and muscle contraction in zebrafish embryos

Xungang Tan*†, Josep Rotllant*, Huiqing Li*, Patrick De Deyne‡, and Shao Jun Du*†§

*Center of Marine Biotechnology, University of Maryland Biotechnology Institute, Baltimore, MD 21202; and ‡Department of Physical Therapy and Rehabilitation Science, University of Maryland School of Medicine, Baltimore, MD 21201

Edited by Eric N. Olson, University of Texas Southwestern Medical Center, Dallas, TX, and approved December 19, 2005 (received for review November 3, 2005)

Histone modification has emerged as a fundamental mechanism for control of gene expression and cell differentiation. Recent studies suggest that SmyD1, a novo SET domain-containing protein, may play a critical role in cardiac muscle differentiation. However, its role in skeletal muscle development and its mechanism of actions remains elusive. Here we report that SmyD1a and SmyD1b, generated by alternative splicing of *SmyD1* gene, are histone methyltransferases that play a key role in skeletal and cardiac muscle contraction. SmyD1a and SmyD1b are specifically expressed in skeletal and cardiac muscles of zebrafish embryos. Knockdown of SmyD1a and SmyD1b expression by morpholino antisense oligos resulted in malfunction of skeletal and cardiac muscles. The SmyD1 morphant embryos (embryos injected with morpholino oligos) could not swim and had no heartbeat. Myofibril organization in the morphant embryos was severely disrupted. The affected myofibers appeared as immature fibers with centrally located nuclei. Together, these data indicate that SmyD1a and SmyD1b are histone methyltransferases and play a critical role in myofibril organization during myofiber maturation.

skeletal muscle | sarcomere | myofiber maturation | cardiac muscle | transgenic fish

The development of skeletal muscles involves a series of events including specification, differentiation, and maturation. During myogenesis, multipotential mesoderm cells are specified to become myoblasts, which ultimately differentiate into matured myofibers that contain highly organized sarcomeres responsible for muscle contraction (1–4). Histone modification plays an important role in muscle-specific gene expression and muscle cell differentiation (5–9). Histone acetylation, catalyzed by histone acetyltransferase (HAT), results in chromatin relaxation and transcriptional activation. Histone deacetylation catalyzed by histone deacetylases (HDACs), in contrast, antagonizes the activity of HAT and represses transcription. HDACs block myogenesis by associating with and inhibiting the activity of MEF2 transcription factor (7). Histone methylation, another form of modification, is involved in both transcription activation and repression.

Histone methylation is carried out by a unique class of enzymes that contain the SET domain, which methylates histones H3 or H4 (10–13). Histone lysine methylation is a relatively stable modification that correlates with transcription inactivation (H3-K4, H3-K36, and H3-K79) or with transcriptionally repressed chromatin (H3-K7, H3-K27, and H4-K20) (14–18). In the past few years, >50 SET domain-containing proteins have been identified (19). They are involved in transcriptional regulation and various cellular processes including cell differentiation, proliferation, chromatin stability, and cell transformation.

SmyD1, also known as skm-Bop, represents a recently identified SET domain-containing protein that is specifically expressed in skeletal and cardiac muscles (20, 21). Targeted deletion of SmyD1 in mice disrupted maturation of cardiomyocytes and formation of the right ventricle (22). SmyD1 null mutants typically die around embryonic day 10.5 (22). Because of the early embryonic lethality of SmyD1 mutant mice, the knockout studies failed to reveal the functions of SmyD1 in skeletal muscles, even though SmyD1 is strongly expressed in skeletal muscles in mouse embryos.

To determine the function of SmyD1 in skeletal muscles, we analyzed the SmyD1 expression and function in zebrafish embryos. The zebrafish provide many advantages over other systems. First, zebrafish embryos can tolerate absence of blood flow because their oxygen is delivered by diffusion rather than by the cardiovascular system. It is therefore possible to study the skeletal muscle defects in zebrafish embryos with cardiac failure (23). Second, myogenesis in zebrafish embryos begins relatively early in development. By 24 h postfertilization (hpf), functional embryonic myofibers are well developed, and mechanical stimuli induce a wiggle reaction (24). Third, the morpholino (MO) antisense technique can be designed to knockdown specific isoforms of mRNA transcripts generated by alternative splicing, which is difficult to do with the gene knockout approach in mice (25).

We report here the isolation and characterization of the *SmyD1* gene and its functions in zebrafish embryos. We have demonstrated that zebrafish SmyD1a and SmyD1b are histone methyltransferases and play key roles in myofiber maturation and contraction. Molecular and cellular analyses revealed that myofibers in SmyD1 knockdown embryos appeared as immature myofibers with centrally located nuclei and disorganized myofibrils, suggesting that SmyD1 plays a critical role in myofiber maturation and contraction.

Results

Isolation and Characterization of Zebrafish SmyD1a and SmyD1b. The full-length *SmyD1a* and *SmyD1b* cDNAs were cloned by RT-PCR from zebrafish. *SmyD1a* encodes a 486-aa protein, whereas *SmyD1b* encodes a 473-aa protein. SmyD1a contains an extra 13-aa insertion at position 215–227. SmyD1a and SmyD1b were generated by alternative splicing. The 13-aa insertion is encoded by the SmyD1a-specific exon 5 (Fig. 6, which is published as supporting information on the PNAS web site).

Zebrafish SmyD1a and SmyD1b are members of the highly conserved SmyD protein family that contain the conserved MYND and SET functional domains. The MYND domain (codons 47–85; Fig. 6*B*) is a zinc-finger domain, which has been implicated in DNA binding and interaction with HDAC proteins. The SET domain has

Conflict of interest statement: No conflicts declared.

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: HDAC, histone deacetylases; hpf, hours postfertilization; MO, morpholino.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. DQ323979 (SmyD1a) and DQ323980 (SmyD1b)].

†X.T. and S.J.D. contributed equally.

§To whom correspondence should be addressed at: Center of Marine Biotechnology, University of Maryland Biotechnology Institute, 701 East Pratt Street, Baltimore, MD 21202. E-mail: dus@umbi.umd.edu.

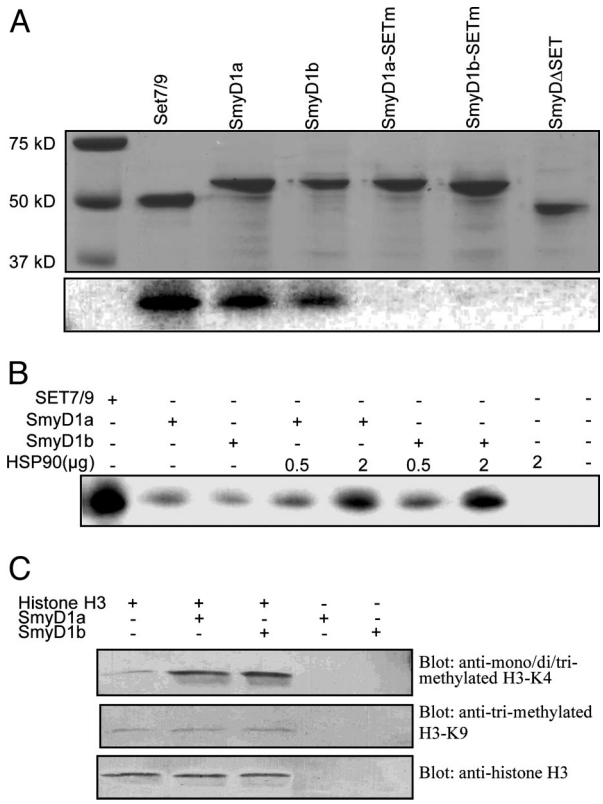

Fig. 1. Histone methyltransferase activity of SmyD1a and SmyD1b. (*A*) Coomassie blue staining showing the purity of the recombinant proteins (1 μg each) used in the HMTase assay. Set7/9, HMTase as positive control; SmyD1a and SmyD1b, purified recombinant SmyD1a and SmyD1b proteins, respectively; SmyD1a-SETm and SmyD1b-SETm, recombinant proteins with four mutations in the SET domain; SmyD1-ΔSET mutant protein with a 75-aa deletion in the SET domain. The MW shift in SmyD1-ΔSET was caused by deletion of the SET domain. The MW of SET7/9 is ≈50,000 Da, which appeared larger than the predicted 40,700 Da. Fluorogram showing tritium-labeled methylated histone H3 in HMTase assay. SmyD1a and SmyD1b showed HMTase activity. Also, 10% of the HMTase assay product was used in the fluorogram of SET7/9. (*B*) Fluorogram showing that HSP90α acts as a cofactor of SmyD1a and SmyD1b in HMTase assay. Addition of 2 μg of HSP90α significantly increased the HMTase activity of SmyD1a and SmyD1b. Also, 20% of the HMTase assay product was used in the fluorogram of SET7/9. (*C*) Specific methylation of histone H3-K4 by SmyD1a and SmyD1b *in vitro*. Methylated H3 proteins were analyzed by Western blot by using antibodies against mono-/di-/tri-methylated H3-K4, tri-methylated H3-K9, or histone H3 proteins. Results showed that SmyD1a or SmyD1b could specifically methylate H3 at lysine 4 but not at lysine 9.

been implicated in histone methylation. The SET domain contains two highly conserved regions ($_{200}$NHXCXPNC$_{207}$ and $_{239}$GEEL/VXXXY$_{246}$) that are critical for interacting with *S*-adenosylmethionine (ref. 10; Fig. 6).

SmyD1a and SmyD1b Are Histone Methyltransferase. SET domain has been implicated in histone methylation (18, 26–28). To determine whether SmyD1a and SmyD1b are histone methyltransferases (HMTase), we performed an *in vitro* histone methylation assay by using recombinant SmyD1a and SmyD1b proteins. The results showed that both SmyD1a and SmyD1b are HMTases that could methylate histone H3 (Fig. 1*A*) but not H4 (data not shown).

To determine whether the SET domain is required for the HMTase activity, we performed a methylation assay by using SmyD1 mutant proteins that lack the SET domain or contain mutations in the SET domain. The conserved sequence ($_{203}$CWPNC$_{207}$) in the SET domain, which is critical for interacting with *S*-adenosylmethionine, was mutated to AAAAA. The results showed that mutating these conserved residues completely abolished the HMTase activity of SmyD1 (Fig. 1*A*), confirming that the SET domain is required for histone methylation.

It has been reported that SmyD3, a SmyD1-related protein, associates with heat shock protein HSP90α, and this association significantly enhances the HMTase activity of SmyD3 *in vitro* (26). To test whether HSP90α could enhance the HMTase activity of SmyD1a and SmyD1b, we added HSP90α in the *in vitro* HMTase assay. Addition of HSP90α significantly enhanced the HMTase activity of SmyD1a and SmyD1b (Fig. 1*B*), suggesting that HSP90α may act as a cofactor in histone methylation.

It was previously reported that SET domain-containing proteins could methylate lysine 4 (K4) or lysine 9 (K9) in histone H3. Methylation of H3-K4 or H3-K9 has the opposite effect on gene transcription (14–18). To determine which lysine residue (K4 or K9) was methylated by SmyD1a and SmyD1b, the methylated H3 proteins were analyzed by Western blot using antibodies against mono-/di-/tri-methylated H3-K4, tri-methylated H3-K9, or histone H3 proteins. Results showed that SmyD1 methylates H3 at lysine 4 but not at lysine 9 (Fig. 1*C*). In addition to K4, other lysine residues such as K27 and K36 could also be methylated in H3, but our data did not rule out the possibility that SmyD1 might methylate these lysine residues.

Temporal and Spatial Expression of SmyD1a and SmyD1b in Zebrafish Embryos. The temporal expression of SmyD1a and SmyD1b was determined by RT-PCR (Fig. 7, which is published as supporting information on the PNAS web site). SmyD1a and SmyD1b exhibited distinct patterns of expression. SmyD1a transcripts were first detected at 6 hpf, and its expression increased significantly during somitogenesis. In contrast, SmyD1b expression came 5 h later than SmyD1a. These data indicate that generation of SmyD1a and SmyD1b by alternative splicing is regulated during development.

The spatial pattern of SmyD1a and SmyD1b expression was determined by whole-mount *in situ* hybridization (Fig. 8, which is published as supporting information on the PNAS web site). Because *SmyD1a* differs from *SmyD1b* by a 39-bp insertion, it was difficult to generate an isoform-specific probe for *in situ* hybridization. Therefore, the spatial patterns of expression of SmyD1a and SmyD1b were determined by using an antisense probe that hybridized with both SmyD1a and SmyD1b mRNA transcripts. The results showed that SmyD1a and/or SmyD1b were expressed in a muscle-specific manner in zebrafish embryos. SmyD1a/b expression was first detected in two lines of adaxial cells flanking the notochord that give rise to slow muscles (Fig. 2*A* and *B*). Later, SmyD1a/b expression was found in lateral regions of the myotome

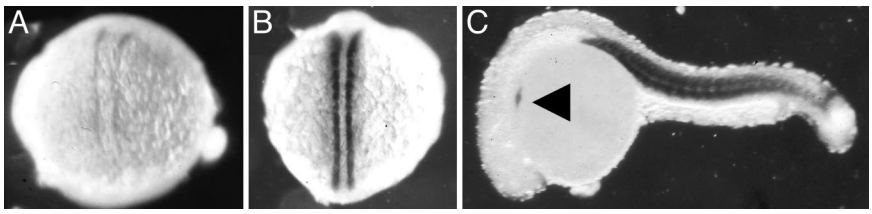

Fig. 2. Temporal and spatial expression of SmyD1a/b in zebrafish embryos. *In situ* hybridization showing the expression patterns of SmyD1a/b by using a dig-labeled antisense probe that hybridizes with both SmyD1a and SmyD1b mRNA transcripts. SmyD1a/b expression was first detected in the adaxial cells flanking the notochord at 10 (*A*) and 12 (*B*) hpf and later in the heart primordium (arrowhead) at 22 hpf (*C*).

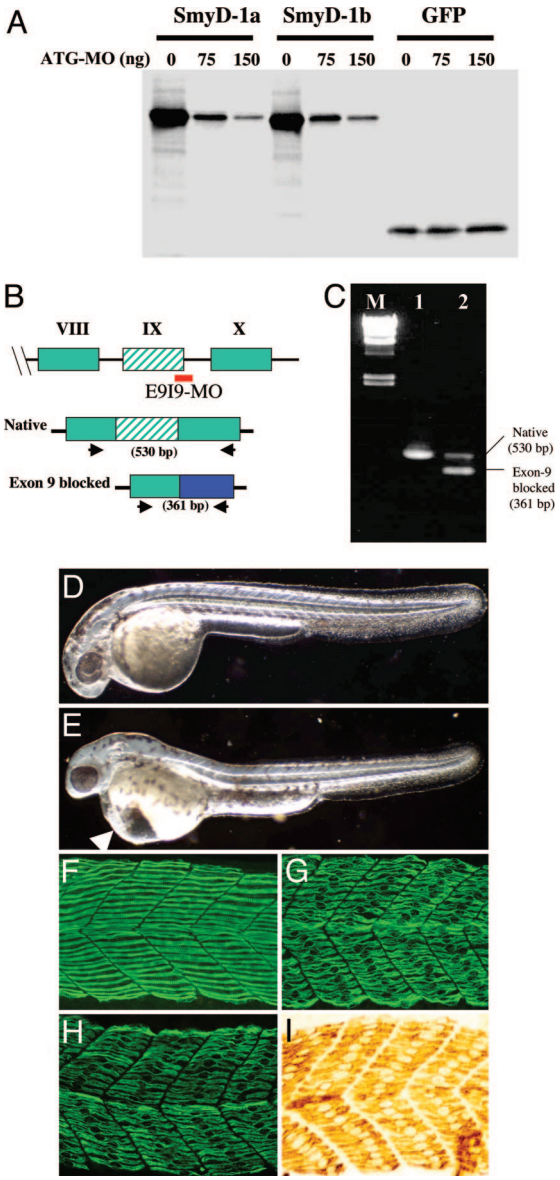

Fig. 3. Knockdown of SmyD1a and SmyD1b expression resulted in cardiac and skeletal muscle defects. (*A*) ATG-MO specifically blocked the expression of SmyD1a or SmyD1b proteins in an *in vitro* transcription and translation assay but had no effect on the GFP translation, even though GFP was cloned in the same expression vector as SmyD1a and SmyD1b DNA constructs. (*B*) Location of the splicing blocker E9I9-MO at the exon 9/intron 9 junction. E9I9-MO blocks the splicing of both SmyD1a and SmyD1b transcripts. (*C*) RT-PCR showing the defective splicing induced by the E9I9-MO splicing blocker. Compared with the PCR results from noninjected embryos where a single band (530 bp) was generated (lane 1), two bands (530 bp and 361 bp) were detected in E9I9-MO-injected embryos (lane 2). The 361-bp band, which was the major PCR product, was a result of defective splicing as shown by DNA sequencing. M, λ/HindIII digested DNA marker. (*D* and *E*) Morphology of control-MO- (*D*) or ATG-MO-injected (*E*) embryos at 48 hpf. ATG-MO induced edemas (arrowhead in *E*) and blood cell accumulation above the yolk sac. (*F–I*) F59 antibody staining showing skeletal muscle defects in SmyD1 knockdown embryos at 24 hpf. Immunostaining by using FTIC-labeled (*F–H*) or peroxidase-labeled (*I*) secondary antibodies. (F) Lateral view of normal myofibril organization in slow muscle fibers at 24 hpf in control-MO-injected embryos. (*G–I*) Defective myofibril organization in ATG-MO- (*G* and *I*) or E9I9-MO-injected (*H*) embryos.

that give rise to fast muscles (Fig. 8). In addition, *SmyD1a/b* was also expressed in heart primordium at 22 hpf (Fig. 2*C*), pectoral fin muscles at 48 hpf, and head muscles at 72 hpf (S.J.D., J.R., and X.T., unpublished work).

Knockdown of SmyD1a and SmyD1b Expression Resulted in Skeletal and Cardiac Muscle Defects. To determine whether or not SmyD1a and SmyD1b function in muscle cell differentiation, we knocked down both SmyD1a and SmyD1b expression in zebrafish embryos by using the translational blocker ATG-MO (Fig. 3*A*). The ATG-MO was injected into zebrafish embryos, and the injected embryos were examined morphologically for 4–5 days after the injection. Although the morphant embryos appeared morphologically normal (Fig. 3*E*), two striking phenotypes were observed. In phenotype one, the morphant embryos (98%, $n = 738$) could not swim and failed to respond to touch. In phenotype two, the morphant embryos did not have a heartbeat, even though the heart was clearly formed despite SmyD1 knockdown (Table 2 and Movies 1 and 2, which are published as supporting information on the PNAS web site). The morphant embryos exhibited clear edema on day 2 or day 3 (Fig. 3*E*) and died at day 5.

To confirm the specificity of these phenotypes, a splicing blocker, E9I9-MO, was injected into zebrafish embryos (Fig. 3*B*). Injection of E9I9-MO caused defective splicing of both SmyD1a and SmyD1b RNA (Fig. 3*C*). Defective splicing of SmyD1a and SmyD1b resulted in a reading-frame shift and led to production of mutant proteins without the highly conserved C-terminal region. E9I9-MO-injected embryos (98.5%, $n = 485$) showed identical muscle defects as ATG-MO-injected embryos, confirming the specificity of SmyD1 knockdown phenotype.

Knockdown of SmyD1a and SmyD1b Expression Disrupted Myofibril Organization. To determine which step of muscle development was affected by SmyD1 knockdown, SmyD1 morphant embryos were analyzed for myoblast specification, differentiation, and maturation by using several molecular and cellular markers. Expression of myogenic markers and formation of slow and fast muscles appeared normal in ATG-MO- or E9I9-MO-injected embryos (Fig. 9, which is published as supporting information on the PNAS web site). These data indicated that SmyD1 was not required for myoblast specification and early differentiation of slow and fast muscles.

To determine whether blocking SmyD1 might disrupt myofiber maturation, ATG-MO- or E9I9-MO-injected embryos were examined for myofibril organization and sarcomere formation by immunostaining using anti-myosin antibody F59. The results indicated that myofibril alignment in slow muscles was highly disorganized in SmyD1 knockdown embryos and formation of sarcomeres appeared significantly reduced at 24 hpf (Fig. 3 *G–I*). Moreover, huge vacuoles representing the nuclei were located in the central region of the myofibers (Fig. 3 *G–I*).

To further characterize the skeletal muscle defect, SmyD1 morphant embryos were analyzed by immunostaining by using antibodies against titin, nebulin, or myosin. Expression of these myofiber proteins appeared normal (data not shown). To characterize the muscle defect at the subcellular level, we next analyzed the morphant embryos by thin section and electronic microscopy (EM). The results showed that myofibrils were highly disorganized in ATG-MO-injected embryos, and sarcomere formation was significantly reduced to small patches (Fig. 4 *C* and *F*). EM analyses showed tightly bundled, hexagonal arrays of thick and thin filaments in a myofiber of the control-MO-injected embryo (Fig. 4*G*) but loosely scattered filaments in a myofiber of the ATG-MO-injected embryos (Fig. 4*H*). Moreover, centrally located huge nuclei were found in the center of the affected myofibers in contrast to the peripheral localization in mature fibers (Fig. 4 *B* and *D*). The central placement of myonuclei is characteristic of newly formed immature myofibers during embryonic development. Together, these data indicate SmyD1 is probably required for myofibril organization during myofiber maturation.

Rescue of Skeletal and Cardiac Muscle Defects by SmyD1a or SmyD1b Minigene. SmyD1a and SmyD1b are different isoforms generated by alternative splicing. To determine whether or not SmyD1a and

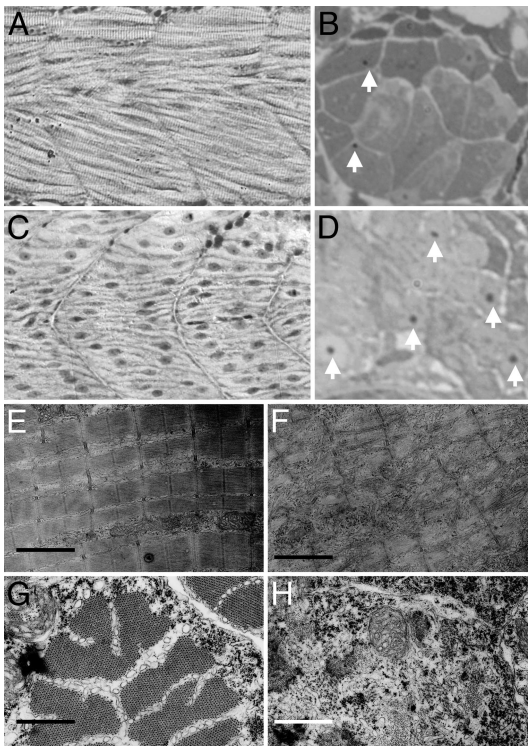

Fig. 4. Myofibril organization is disrupted in SmyD1a/b knockdown embryos. (*A*, *C*, *E*, and *F*) Longitudinal sections showing highly organized myofibrils in control-MO-injected embryo (*A* and *E*) or disorganized myofibrils in ATG-MO-injected embryos (*C* and *F*) at 48 hpf. (*A* and *C*) Toluidine staining on plastic sections. (*E* and *F*) Photographed by using transmission electromicroscopy. (Scale bars: 2 μm.) (*B* and *D*) Toluidine staining on cross sections showing periferal nuclear localization of myofibers in control-MO-injected embryo (*B*) or centronuclear localization in ATG-MO-injected embryo (*D*) at 48 hpf. Arrows in *B* and *D* indicate nuclei. (*G* and *H*) Transmission electromicroscopy showing tightly bundled, hexagonal arrays of thick and thin filaments in a myofiber of control-MO-injected embryo (*G*) or loosely scattered filaments in a myofiber of ATG-MO-injected embryos (*H*) at 48 hpf. (Scale bars: 1.4 μm.)

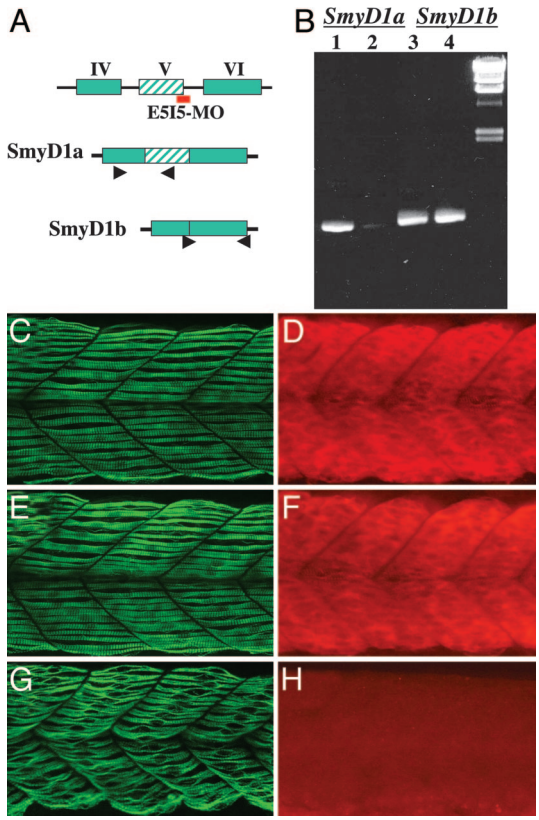

Fig. 5. Analysis of SmyD1a or SmyD1b-specific functions by combination of knockdown and transgenic approaches. (*A*) Diagram showing the location of SmyD1a-specific E5I5-MO-splicing blocker at the exon 5/intron 5 junction. E5I5-MO specifically blocks the splicing of SmyD1a because exon 5 is not used in SmyD1b. (*B*) RT-PCR showing specific knockdown of SmyD1a by E5I5-MO-splicing blocker. Lanes 1 and 3, SmyD1a or SmyD1b expression, respectively, in control-MO-injected embryos; lanes 2 and 4, SmyD1a or SmyD1b expression, respectively, in E5I5-MO-injected embryos. (*C–H*) Double staining showing the rescue of skeletal muscle defects by SmyD1a or SmyD1b minigenes in E9I9-MO-injected embryos at 24 hpf. (*C*, *E*, and *G*) F59 antibody staining showing normal myofibril alignment in SmyD1a (*C*) or SmyD1b (*E*) transgenic embryos injected with E9I9-MO or defective myofibril organization in nontransgenic embryos (*G*) injected with E9I9-MO. (*D*, *F*, and *H*) Anti-myc-tag antibody staining showing the expression of myc-tagged SmyD1a (*D*) or SmyD1b (*F*) in transgenic embryos, or nontransgenic control (*H*).

SmyD1b have distinct functions, we knocked down SmyD1a or SmyD1b individually in zebrafish embryos. SmyD1a was specifically knocked down by using a splicing blocker (E5I5-MO) targeted to the SmyD1a-specific exon 5 (Fig. 5*A*). Defective splicing of exon 5 resulted in production of mRNA corresponding to SmyD1b (Fig. 5*B*). Knockdown of SmyD1a alone did not affect muscle development. SmyD1a morphant embryos (90%, $n = 239$) could still swim and had normal heart contraction. Immunostaining showed that knockdown SmyD1a morphants had normal myofibril alignment (Fig. 10, which is published as supporting information on the PNAS web site). These data indicate that SmyD1a and SmyD1b might have redundant functions, or alternatively, SmyD1b may be more critical for myofiber maturation.

To clarify the above hypothesis, we next specifically knocked down SmyD1b expression in zebrafish embryos. Unlike SmyD1a, no morpholino oligo could be designed to specifically knockdown SmyD1b; therefore, we combined a knockdown approach with a transgenic rescue approach. In this approach, we first generated transgenic zebrafish that expressed a myc-tagged SmyD1a minigene driven by the *smyd1* promoter. Expression of the minigene recapitulated that of the endogenous *SmyD1* gene (Fig. 10). Because the minigene was constructed by using the SmyD1a cDNA, which does not require splicing for expression, injection of E9I9-MO splicing blocker had no effect on the expression of the SmyD1a minigene (Fig. 5*D*). Consequently, injection of E9I9-MO into SmyD1a transgenic embryos specifically blocked SmyD1b expression. SmyD1a transgenic embryos injected with E9I9-MO showed normal heartbeat and locomotion and no sign of edema (Fig. 10; Table 1). Using the same transgenic approach, we have created SmyD1b transgenic fish (Fig. 10). Knockdown of SmyD1a expression had no effect on skeletal and cardiac muscle contraction (Table 1).

To confirm that expression of SmyD1a or SmyD1b minigenes could rescue the skeletal muscle defects from E9I9 injection, SmyD1a or SmyD1b transgenic embryos injected with E9I9 were analyzed by double staining with anti-myc or F59 antibodies (Fig. 5 *C–F*). Immunostaining revealed normal myofibril organization in SmyD1a or SmyD1b transgenic embryos in which endogenous SmyD1a and SmyD1b were knocked down (Fig. 5 *C* and *E*). Together, these data suggest that SmyD1a and SmyD1b have redundant functions in the control of myofiber organization and knockdown of both SmyD1a and SmyD1b are required to completely inhibit SmyD1 function.

Histone Methyltransferase Activity of SmyD1a and SmyD1b Is Essential for Their Biological Functions in Muscle Cell Differentiation. To determine whether HMTase activity is required for SmyD1a and

Table 1. Rescue of skeletal and cardiac muscle defects by SmyD1a or SmyD1b minigenes

Embryo	No. of embryos	No. with muscle contraction	No. without muscle contraction	Rescue, %
SmyD1a transgenic embryos + E9I9-MO (5 ng)	27	27	0	100
Nontransgenic embryos + E9I9-MO (5 ng)	32	0	32	0
SmyD1b transgenic embryos + E9I9-MO (5 ng)	39	38	1*	97
Nontransgenic embryos + E9I9-MO (5 ng)	38	0	38	0

Mature heterozygous F1 transgenic fish expressing SmyD1a or SmyD1b minigenes were crossed with wild-type nontransgenic fish. The resulting embryos were injected with 5 ng of E9I9-MO. Embryos showing skeletal and cardiac muscle defects were separated from normal embryos at 24 hpf. The two groups of embryos were analyzed by anti-myc-antibody staining that recognized the myc-tagged SmyD1a or myc-tagged SmyD1b proteins. All embryos that could swim and had a normal heartbeat (with muscle contraction) were transgenic embryos, whereas the embryos without muscle contraction were nontransgenic embryos.
*This particular transgenic embryo was severely deformed, presumably because of physical damage from the microinjection.

SmyD1b function in myofiber maturation, we performed a rescue experiment by using DNA constructs encoding wild-type SmyD1a or SmyD1b proteins or their mutants that lack the HMTase activity. The DNA construct was coinjected with E9I9-MO into zebrafish embryos. The results showed that expression of SmyD1a or SmyD1b mutant proteins without HMTase activity failed to rescue myofiber defects (Fig. 11 and Table 3, which are published as supporting information on the PNAS web site). By contrast, expression of wild-type SmyD1a or SmyD1b could rescue the myofiber defects in E9I9-MO-injected embryos (Fig. 11 and Table 3). The rescue was clearly mosaic, as predicated from the transient expression by DNA injection. Moreover, the rescue appeared to be cell autonomous, because double staining revealed that the myofiber that appeared normal was the only fiber that expressed the myc-tagged SmyD1a or SmyD1b proteins (Fig. 11). Together, these data indicate that the HMTase activity of SmyD1a and SmyD1b is essential for their functions in myofiber maturation, arguing that SmyD1a and SmyD1b may control myofiber maturation through histone methylation.

Discussion

In this study, we have characterized the expression and functions of SmyD1a/b in zebrafish embryos and demonstrated that SmyD1a and SmyD1b are histone methyltransferases that play key roles in myofiber maturation. Knockdown of SmyD1a/b expression resulted in defective myofibers with disorganized myofibril alignment and a centrally located nucleus, a characteristic of immature myofibers. Together, this study demonstrates that histone methylation is involved in myofiber maturation.

SmyD1a and SmyD1b Have Redundant Function. We used a knockdown approach together with a transgenic approach to specifically inhibit SmyD1a or SmyD1b expression. To our knowledge, this publication is the first report of such an approach being used to determine functions of specific gene isoforms in zebrafish embryos. We showed that inhibition of SmyD1a or SmyD1b expression alone did not affect locomotion or heart contraction. In contrast, blocking both SmyD1a and SmyD1b expression completely inhibited muscle contraction and myofibril alignment, suggesting that SmyD1a and SmyD1b may share some overlapping functions. However, this study could not rule out the possibility that SmyD1a and SmyD1b may have other specific functions that could not be revealed by our analyses in this study.

SmyD1a and SmyD1b Are Histone Methyltransferases. Histone methylation cooperates with DNA methylation to regulate gene expression and to establish long-term cell identity (29). Methylation of lysine 4 of histone H3 is linked to transcriptional activation, whereas methylation of lysine 9 in H3 is tightly associated with gene repression (14–18). Methylation of histone H3 lysine 9 by SUV39H1 creates a binding site for heterochromatin protein 1 (HP1) (30, 31). HP1 associates with a variety of transcriptional repressors and thereby provides a mechanism for widespread silencing of gene expression (32–35). It has been demonstrated that HP1 associates with HDACs to silence MEF2 target genes during myogenesis (36). We have established in this study that SmyD1a/b methylate lysine 4, but not lysine 9, of histone H3, suggesting that SmyD1a/b may be involved in gene activation. However, we cannot rule out the possibility that SmyD1a/b may also methylate other lysine residues in H3 or other proteins that may complicate the functions and regulation of gene expression by SmyD1a/b.

Histone Methylation and Myofiber Maturation. SmyD1a/b may function downstream of myogenic regulatory factors in controlling gene expression and muscle cell differentiation. It has been shown that SmyD1 expression is the directly regulated by MyoD, myogenin, and Mef2 (37, 38). Expression of SmyD1 could, in turn, activate expression of other genes through histone modification. It has been shown that expression of *hand2* in cardiac muscle requires SmyD1 (22). Hamamoto *et al.* (26) showed that SmyD3, a SmyD1 related protein, directly binds DNA to control the expression of genes important for development. The target genes of SmyD1, however, have yet to be identified.

In recent years, several publications have strongly implicated SET domain-containing proteins in muscle cell differentiation. Caretti and colleagues (39) reported that Polycomb protein Ezh2, a SET domain-containing protein, regulates muscle gene expression and skeletal muscle differentiation by transcriptional repression. Baxendale and colleagues (40) have demonstrated that Blimp-1, another SET-domain protein, controls slow muscle differentiation in zebrafish embryos. In the Blimp-1 mutant (u-boot), arrangement of the slow myofibrils appears dramatically altered (41). We demonstrated in this study that SmyD1 mutant constructs without HMTase activity were unable to rescue myofiber defects in SmyD1 knockdown embryos, suggesting that the HMTase activity is required for its function in myofiber maturation. However, we can also speculate that mutating the SET domain might alter SmyD1 folding, automethylation, or binding with other proteins that are required for its function in myofiber maturation.

Cofactors of SmyD1a and SmyD1b. We have demonstrated that HSP90α enhanced the HMTase activity of SmyD1. Members of the HSP90 family modulate the activity of signaling molecules and transcription factors (42–44). Strong hsp90α expression has been identified in developing somites of zebrafish and chicken embryos (45–47). HSP90α may control muscle cell differentiation by facilitating histone methylation. This hypothesis is consistent with previous findings that HSP90α plays a role in muscle cell differentiation (48–50). In addition to HSP90, SmyD1 may interact with other proteins to regulate gene expression and muscle cell differentiation. SmyD1 interacts with skNAC, a heart and muscle-specific transcription factor involved in muscle regeneration (51–53). Moreover, SmyD1 represses gene transcription in an HDAC-dependent manner (22). Thus, SmyD1 may provide an efficient mechanism to

couple histone methylation with deacetylation in the control of gene expression and muscle cell differentiation.

Materials and Methods

Synthesis of Morpholino Antisense Oligos for Translation and Splicing Blockers. Morpholino antisense oligos were synthesized by Gene Tools (Carvalis, OR). The translation blocker (ATG-MO) was based on a sequence near the ATG (in bold) start site. The splicing blocker (E9I9-MO) was based on the sequence at the exon 9 and intron 9 junctions. E5I5-MO splicing blocker was based on the sequence at the exon 5 and intron 5 junctions: ATG-MO: 5′-ACTTCCAAACTC**CAT**TCTGGATC-3′; E9I9-MO: 5′-CGT-CACCTCTAGGTCTTTAGTGATG-3′; and E5I5-MO: 5′-GATCTGAAAACCCACCTCTTCTGAG-3′.

Morpholino Microinjection in Zebrafish Embryos. Morpholino antisense oligos were dissolved in 1X Danieau buffer (25) to a final concentration of 0.5 mM or 1 mM. Next, ≈1–2 nl (5–10 ng) was injected into each embryo. For coinjection, equal volumes of E9I9-MO (1 mM) and DNA construct (100 μg/ml) was mixed for microinjection.

Transcription and Translation Assay and *in Vitro* Analysis of ATG-MO Blocker. The activity of ATG-MO translation blocker was tested by *in vitro* transcription and translation assay by using the manufacturer's kit (Promega). Seventy nanograms or 150 ng of ATG-MO antisense was added in the transcription and translation assay reaction containing 1 μg of *cmv-SmyD1a*^{myc} or *cmv-SmyD1b* ^{myc} or *cmv-GFP* plasmid DNA (see *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site, for detailed construction). The protein products were analyzed on a 12% SDS/PAGE.

Production of *smyd1-SmyD1a*^{myc} and *smyd1-SmyD1b*^{myc} Transgenic Zebrafish. *smyd1-SmyD1a*^{myc} and *smyd1-SmyD1b*^{myc} minigenes were constructed by using cDNA encoding the myc-tagged SmyD1a or myc-tagged SmyD1b cloned after the 5.3-kb zebrafish *smyd1* promoter and its 5′ flanking sequence. *smyd1-SmyD1a*^{myc} and *smyd1-SmyD1b*^{myc} DNA constructs were linearized with SalI and microinjected into zebrafish embryos as described (54). Germ-line transgenic founders were screened by whole-mount anti-myc tag antibody staining on F1 embryos at 24 hpf. Adult F1 transgenic fish were identified by PCR by using DNA from caudal fin.

Histone Methyltrasferase Assay *in Vitro*. The histone methyltrasferase assay was carried out as described by Hamamoto *et al.* (26) with some modifications. Briefly, recombinant proteins (*Supporting Materials and Methods*) of SmyD1a or SmyD1b (1 μg) or their SET-domain mutants (SmyD1a-SETm, SmyD1b-SETm, and SmyD1-ΔSET) were incubated with 1 μg of recombinant histone H3 or H4 proteins (Upstate) and 2 μCi (1 Ci = 37 GBq) *S*-adenosylmethionine (SAM; Amersham Pharmacia Biosciences) in a mixture of 40-μl reaction buffer (50 mM Tris·HCl, pH 8.5/100 mM NaCl/10 mM DTT) for 3 h at 30°C. SET7/9 (Alexis Biochemicals, San Diego) was used as positive control. To determine whether HSP90α acts as a cofactor, 0.5–2 μg of human HSP90α (Calbiochem) was added to the reaction. To examine H3-K4 methyltranferase activity, histone H3 protein (Upstate Biotechnology, Lake Placid, NY) was incubated with SAM in the presence or absence of recombinant SmyD1a or SmyD1b proteins at 30°C for 3 h. The proteins were analyzed by Western blotting by using antibodies (Upstate Biotechnology) against mono-/di-/tri-methylated H3-K4, tri-methylated H3-K9, or H3.

We thank John Stubblefield and Jennifer Petering for editing and Donald Grant for assistance in transgenic fish screening. This work was supported by National Institutes of Health Grant R01GM58537, Maryland Sea Grant, and Binational Agricultural Research and Development Fund Grant (to S.J.D.), and by a Fulbright-Ministerio de Educacion Cultura y Deportes Grant (Fu-2004–0962) (to J.R.). This publication is contribution no. 05-138 from the Center of Marine Biotechnology, University of Maryland Biotechnology Institute.

1. Buckingham, M. (2001) *Curr. Opin. Genet. Dev.* **11,** 440–448.
2. Brent, E. & Tabin, C. J. (2002) *Curr. Opin. Genet. Dev.* **12,** 548–557.
3. Pownall, M. E., Gustafsson, M. K. & Emerson, C. P., Jr. (2002) *Annu. Rev. Cell Dev. Biol.* **18,** 747–783.
4. Parker, M. H., Seale, P. & Rudnicki, M. A. (2003) *Nat. Rev. Genet.* **4,** 497–507.
5. Lee, H., Habas, R. & Abate-Shen, C. (2004) *Science* **304,** 1675–1678.
6. McKinsey, T. A., Zhang, C. L. & Olson, E. N. (2001) *Curr. Opin. Genet. Dev.* **11,** 497–504.
7. McKinsey, T. A., Zhang, C. L. & Olson, E. N. (2002) *Curr. Opin. Cell Biol.* **14,** 763–772.
8. Rupp, R. A., Singhal, N. & Veenstra, G. J. (2002) *Eur. J. Biochem.* **269,** 2294–2299.
9. Cirillo, L. & Zaret, K. (2004) *Science* **304,** 1607–1609.
10. Rea, S., Eisenhaber, F., O'Carroll, D., Strahl, B. D., Sun, Z. W., Schmid, M., Opravil, S., Mechtler, K., Ponting, C. P., Allis, C. D. & Jenuwein, T. (2000) *Nature* **406,** 593–599.
11. Cheng, X., Collins, R. E. & Zhang, X. (2005) *Annu. Rev. Biophys. Biomol. Struct.* **34,** 267–294.
12. Nishioka, K., Rice, J. C., Sarma, K., Erdjument-Bromage, H., Werner, J., Wang, Y., Chuikov, S., Valenzuela, P., Tempst P., Steward, R., *et al.* (2002) *Mol. Cell* **9,** 1201–1213.
13. Karachentsev D., Sarma K., Reinberg D. & Steward, R. (2005) *Genes Dev.* **19,** 431–435.
14. Santos-Rosa, H., Schneider, R., Bannister, A. J., Sherriff, J., Bernstein, B. E., Emre, N. C., Schreiber, S. L., Mellor, J. & Kouzarides, T. (2002) *Nature* **419,** 407–411.
15. Strahl, B. D., Ohba, R., Cook, R. G. & Allis, C. D. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 14967–14972.
16. Rice, J. C. & Allis, C. D. (2001) *Nature* **414,** 258–261.
17. van Holde, K. E. (1988) *Chromatin* (Springer, New York).
18. Zhang, Y. & Reinberg, D. (2001) *Genes Dev.* **15,** 2343–2360.
19. Kubicek, S. & Jenuwein, T. (2004) *Cell* **119,** 903–906.
20. Hwang, I. & Gottlieb, P. D. (1997) *J. Immunol.* **158,** 1165–1174.
21. Hwang, I. & Gottlieb, P. D. (1995) *Immunogenetics* **42,** 353–361.
22. Gottlieb, P. D., Pierce, S. A., Sims, R. J., Yamagishi, H., Weihe, E. K., Harriss, J. V., Maika, S. D., Kuziel, W. A., King, H. L. Olson, E. N., *et al.* (2002) *Nat. Genet.* **31,** 25–32.
23. Granato, M., van Eeden, F. J., Schach, U., Trowe, T., Brand, M., Furutani-Seiki, M., Haffter, P., Hammerschmidt, M., Heisenberg, C. P., Jiang, Y. J., *et al.* (1996) *Development (Cambridge, U.K.)* **123,** 399–413.
24. Felsenfeld, A. L., Walker, C., Westerfield, M., Kimmel, C. & Streisinger, G. (1990) *Development (Cambridge, U.K.)* **108,** 443–459.
25. Ekker, S. C. & Larson, J. D. (2001) *Genesis* **30,** 89–93.
26. Hamamoto, R., Furukawa, Y., Morita, M., Iimura, Y., Silva, F. P., Li, M., Yagyu, R. & Nakamura, Y. (2004) *Nat. Cell Biol.* **6,** 731–740.
27. Yin, Y., Liu, C., Tsai, S. N., Zhou, B., Ngai, S. M. & Zhu, G. (2005) *J. Biol. Chem.* **280,** 30025–30031.
28. Lachner, M. & Jenuwein T. (2002) *Curr. Opin. Cell Biol.* **14,** 286–298.
29. Sims, R. J., III, & Reinberg, D. (2004) *Nat. Cell Biol.* **6,** 685–687.
30. Lachner, M., O'Carroll, D., Rea, S., Mechtler, K. & Jenuwein, T. (2001) *Nature* **410,** 116–120.
31. Bannister, A. J., Zegerman, P., Partridge, J. F., Miska, E. A., Thomas, J. O., Allshire, R. C. & Kouzarides, T. (2001) *Nature* **410,** 120–124.
32. Lechner, M. S., Begg, G. E., Speicher, D. W. & Rauscher, F. J., III (2000) *Mol. Cell. Biol.* **20,** 6449–6465.
33. Nielsen, A. L., Ortiz, J. A., You, J., Oulad-Abdelghani, M., Khechumian, R., Gansmuller, A., Chambon, P. & Losson, R. (1999) *EMBO J.* **18,** 6385–6395.
34. Nielsen, A. L., Oulad-Abdelghani, M., Ortiz, J. A., Remboutsika, E., Chambon, P. & Losson, R. (2001) *Mol. Cell* **7,** 729–739.
35. Ryan, R. F., Schultz, D. C., Ayyanathan, K., Singh, P. B., Friedman, J. R., Fredericks, W. J. & Rauscher, F. J., III (1999) *Mol. Cell. Biol.* **19,** 4366–4378.
36. Zhang, C. L., McKinsey, T. A. & Olson, E. N. (2002) *Mol. Cell. Biol.* **22,** 7302–7312.
37. Blais, A., Tsikitis, M., Acosta-Alvear, D., Sharan, R., Kluger, Y. & Dynlacht, B. D. (2005) *Genes Dev.* **19,** 553–569.
38. Phan, D., Rasmussen, T. L., Nakagawa, O., McAnally, J., Gottlieb, P. D., Tucker, P. W., Richardson, J. A., Bassel-Duby, R. & Olson, E. N. (2005) *Development (Cambridge, U.K.)* **132,** 2669–2678.
39. Caretti, G., Di Padova, M., Micales, B., Lyons, G. E. & Sartorelli, V. (2004) *Genes Dev.* **18,** 2627–2638.
40. Baxendale, S., Davison, C., Muxworthy, C., Wolff, C., Ingham, P. W. & Roy, S. (2004) *Nat. Genet.* **36,** 88–93.
41. Roy, S., Wolff, C. & Ingham, P. W. (2001) *Genes Dev.* **15,** 1563–1576.
42. Catelli, M. G., Binart, N., Jung-Testas, I., Renoir, J. M., Baulieu, E. E., Feramisco, J. R. & Welch, W. J. (1985) *EMBO J.* **4,** 3131–3135.
43. Pratt, W. B. (1992) *BioEssays* **14,** 841–848.
44. Shaknovich, R., Shue, G. & Kohtz, D. S. (1992) *Mol. Cell. Biol.* **12,** 5059–5068.
45. Sass, J. B., Weinberg, E. S. & Krone, P. H. (1996) *Mech. Dev.* **54,** 195–204.
46. Sass, J. B. & Krone, P. H. (1997) *Exp. Cell Res.* **233,** 391–394.
47. Sass, J. B., Martin, C. C. & Krone, P. H. (1999) *Int. J. Dev. Biol.* **43,** 835–838.
48. Lele, Z., Hartson, S. D., Martin, C. C., Whitesell, L., Matts, R. L. & Krone, P. H. (1999) *Dev. Biol.* **210,** 56–70.
49. Shue, G. & Kohtz, D. S. (1994) *J. Biol. Chem.* **269,** 2707–2711.
50. Miyata, Y. & Yahara, I. (1995) *Biochemistry* **34,** 8123–8129.
51. Sims, R. J., III, Weihe, E. K., Zhu, L., O'Malley, S., Harriss, J. V. & Gottlieb, P. D. (2002) *J. Biol. Chem.* **277,** 26524–26529.
52. Munz, B., Wiedmann, M., Lochmuller, H. & Werner, S. (1999) *J. Biol. Chem.* **274,** 13305–13310.
53. Yotov, W. V. & St. Arnaud, R. (1996) *Genes Dev.* **10,** 1763–1772.
54. Du, S. J. & Dienhart, M. (2001) *Dev. Dyn.* **222,** 655–666.